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(54) Title: IDENTIFICATION OF INHIBITORS OF PROTEIN TYROSINE KINASE 2

(57) Abstract

Assays for compounds which bind to or modulate the activity of Protein Tyrosine Kinase 2 are given. These ligands are useful in treating osteoporosis and/or inflammation.

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TITLE OF THE INVENTION

IDENTIFICATION OF INHIBITORS OF PROTEIN TYROSINE KINASE 2

BRIEF DESCRIPTION OF THE INVENTION

This invention is directed to a method of identifying compounds which bind to and/or modulate the activity of the enzyme Protein Tyrosine Kinase (PYK2). Such compounds are useful in the prevention and treatment of osteoporosis and inflammation states.

BACKGROUND OF THE INVENTION

Protein Tyrosine Kinase 2 (PYK2), also referred to as Cell Adhesion Kinase β (CAKβ), and Related Adhesion Focal Tyrosine Kinase (RAFTK) is a recently described member of the focal adhesion kinase family. See Avraham, et al., 1995 *J. Biol. Chem.* 270:27742-27751; Lev, et al., 1995 *Nature* 376:737-745; and Sasaki, et al., 1995 *J. Biol. Chem.* 270:21206-21219. PYK2 has been cloned from various sources, including mouse, rat and human brain libraries, and the human megakaryocytic CMK cell line.

Monocyte-macrophages are migratory phagocytic cells which play an important role in immunity and inflammation, in part due to their capacity to secrete bioactive molecules. Macrophage function is regulated to a large degree by adhesion to surrounding extracellular matrix (ECM) and by responses to specific cytokines. Monocyte/macrophage adhesion, chemotaxis and phagocytosis are mainly mediated by β_2 integrins, whose members are classified according to the α chain as $\alpha_L\beta_2$ (LFA-1; CD11a/CD18), $\alpha_M\beta_2$ (Mac-1; CR3; CD11b/CD18) and $\alpha_X\beta_2$ (gp150,95; CD11c/CD18). The adhesion of monocytes is also influenced by members of the β_1 integrins, particularly $\alpha_4\beta_1$ (VLA4) and the α_V-associated integrins.

In vitro, most cells adhere to ECM via focal adhesion contacts. However, monocytic cells adhere to substrate through dot-shaped contact sites named "podosomes". Like focal adhesions, podosomes are regions of the cell surface where the plasma membrane is in close contact with the underlying substrate.

Podosomes have been detected in many transformed cells but are also extensively present in spreading macrophages and osteoclasts.

It would be desirable to identify compounds which would inhibit the formation of podosomes, as these compounds would be potential anti-inflammation and/or anti-osteoporosis agents. However, to date there is no assay for identifying such compounds.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to a method of identifying a compound which binds to and/or modulates the activity of Protein Tyrosine Kinase 2 (PYK2) comprising contacting the compound and PYK2 and determining if binding has occurred. Further, if binding has occurred, the activity of the bound PYK2 may be compared to activity of PYK2 which is not bound to the compound to determine if the compound modulates PYK2 activity. Murine PYK2 cDNA is set forth in Figure 8 (SEQ ID NO:5). A deduced murine PYK2 protein is also set forth in Figure 8 (SEQ ID NO:6).

This invention also relates to a method for identifying compounds which inhibit the formation of podosomes in macrophage cells comprising: contacting the compound with protein tyrosine kinase 2 (PYK2) and determining if the compound inhibits PYK2 activity.

This invention also relates to a method of identifying a compound which prevents monocyte adhesion to a substrate comprising: contacting the compound with protein tyrosine kinase 2 (PYK2) and determining if the compound inhibits PYK2 activity.

Another aspect of this invention is a method of identifying a compound which inhibits osteoclast mobility comprising: contacting the compound with protein tyrosine kinase 2 (PYK2) and determining if the compound inhibits PYK2 activity.

A further aspect of this invention is a method of identifying a compound which inhibits a monocytic cell from degrading an extracellular matrix comprising: contacting the compound with protein tyrosine kinase 2 (PYK2) and determining if the compound inhibits PYK2 activity.

The present invention relates to compounds which are identified using the assays of the present invention. The compounds which are identified are useful in the prevention and treatment of osteoporosis, inflammation, and other conditions dependent upon monocyte migration and invasion activities.

The present invention also relates to methods of treating and/or preventing a disease state or condition in a mammal which is mediated by PYK2. The present invention also relates to methods of treating and/or preventing osteoporosis and/or inflammation in mammals by administering an effective amount of the compounds which are identified using the assays of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 is a Northern Blot analysis of PYK2 expression in mouse tissues. The same RNA blot was hybridized first with a specific probe for mouse PYK2 (upper panel) and then stripped and probed for mouse Focal Adhesion Kinase (FAK) (lower panel) as described. RNA size markers are indicated on the right.

FIGURE 2 shows characterization of the polyclonal anti-PYK2 antibodies. Specificity of the anti-PYK2 antibodies was assessed by immunoprecipitation using either anti-PYK2 antiserum (upper panel) or anti-FAK mAb 2A7 (lower panel) from cell extracts either isolated from mock-transfected human embryonic kidney 293 cells (lane 1), or stably transfected with mouse FAK cDNA (lane 2) and mouse PYK2 cDNA (lane 3). The level of expression of each protein was visualized by immunoblot analysis using the respective antibodies. Expression of PYK2 and FAK were examined in parental NIH3T3 cells (wt., lane 4), or in Ras-transformed (lane 5) and in Src-transformed (lane 6) NIH3T3 cells. Protein levels of PYK2 (upper panel) and FAK (lower panel) were detected by immunoprecipitation, followed by immunoblot analysis.

FIGURE 3 shows expression of PYK2 in murine monocyte-macrophages in primary thioglycolate-induced peritoneal macrophages (lane 1), immortalized peritoneal IC-21 macrophages (lane 2), monocyte-macrophage RAW264.7 (lane 3), WEHI-3 (lane 4)

and P388D1 (lane 5) cell lines. Expression of these proteins was also examined in isolated mouse bone marrow cells (lane 6), in bone marrow-derrived M-CSF induced macrophages (lane 7) and bone marrow-derived 1,25(OH)$_2$D$_3$ -induced osteoclast-like cells (lane 8). To visualize the doublet on SDS-PAGE that represents the closely separated forms of PYK2, the immunoblot for the levels of PYK2 in these cells was underdeveloped.

FIGURE 4 demonstrates that cell adhesion stimulates tyrosine phosphorylation of PYK2 in the IC-21 macrophages in suspension or upon attachment to various ECM. Cells detached by trypsinization (lane 1) or replated on tissue-culture plastic in the presence of complete serum (lane 2). Otherwise, cells in suspension were allowed to attached for 20 min on polystyrene dishes coated with 100 μg/ml poly-L-lysine (lane 3), 25 μg/ml fibronectin (FN, lane 4), 10 μg/ml vitronectin (VN, lane 5), 50 μg/ml fibrinogen (FB, lane 6), 25 μg/ml laminin (LN, lane 7), 25 μg/ml collagen type I (COL I, lane 8) and 25 μg/ml collagen type IV (COL IV, lane 9). The blot was first incubated with anti-phosphotyrosine mAb 4G10 (upper panel), then stripped and re-blotted with anti-PYK2 antibodies (lower panel). The arrows indicate two forms of PYK2 based on differences in molecular weight.

FIGURES 5A, 5B, and 5C demonstrate that adhesion-mediated increase in PYK2 tyrosine phosphorylation is time dependent. In Figure 5A, IC-21 cells were attached to tissue culture dishes for 4h in the presence of serum (ON DISH, lane 1) or maintained in suspension for 1h in serum-free condition (OFF DISH, lane 2). Cells were allowed to attach to fibronectin (FN)-coated dishes in serum-free medium for the indicated times (lanes 3-8). Levels of phosphotyrosine (upper panel) and PYK2 protein (lower panel) were determined by immunoblots using anti-P-Tyr 4G10 mAb and anti-PYK2 Ab, respectively.

In Figure 5B, the kinetics of cell adhesion-induced PYK2 tyrosine phosphorylation were also followed by estimating the mean relative tyrosine phosphorylation of the kinase in IC-21 cells attached to fibrinogen (Fb), fibronectin (Fn), vitronectin (Vn) or poly-L-lysine

(Poly-L-Lys). In each case, the specific activity of tyrosine phosphorylatated PYK2 was calculated by normalizing to the protein level. The PYK2 tyrosine phosphorylation in ON DISH (arbitrarily set at 1.0) was used as reference. Bars represent mean and SD from three independent experiments.

Figure 5C shows *in vitro* kinase assays of anti-PYK2 immunocomplexes from IC-21 cells plated on fibronectin (Fn). The bar graph represents the calculated specific activity of total [^{32}P] incorporation into poly(Glu,Tyr) after kinase assays of immunoprecipitated PYK2 complexes, normalized to the protein level as determined by western blots. Again, the activity of PYK2 in ON DISH was arbitrarily set at 1.0, and all numbers represent averages from three experiments.

FIGURE 6A demonstrates that adhesion-induced tyrosine phosphorylation of PYK2 is mediated by integrin $\alpha_M\beta_2$ in macrophages. Expression pattern of integrins in IC-21 macrophages was determined by by flow cytometric analysis. Cells were incubated with the following antibodies: mAb M17/4 (anti-α_L); mAb M1/70 (anti-α_M); mAb HL3 (anti-α_X); mAb M18/2 (anti-β_2); mAb R1-2 (anti-α_4); mAb MFR5 (anti-α_5); mAb H9.2B8 (anti-α_v); and mAb 9EG7 (anti-β_1); followed by incubation with FITC-conjugated goat anti-rat IgG or FITC-cojugated goat anti-hamster IgG. Open peaks represent cells with secondary antibody treatment alone and filled peaks represent cells incubated with anti-integrin antibodies.

Figure 6B shows IC-21 cells which were allowed to attach to fibrinogen in the absence (lane 1) or the presence of blocking antibodies to the following integrin subunits: anti-α_L (lane 2), anti-α_M (lane 3), anti-β_2 (lane 4), anti-α_4 (lane 5), anti-α_5 (lane 6) and anti-β_1 (lane 7). Tyrosine phosphorylation of PYK2 was determined by immunoprecipitation and immunoblot analysis.

Figure 6C shows that relative PYK2 tyrosine phosphorylation was quantitated in cells adhering to fibrinogen (Fb, upper panel), to fibronectin (Fn, middle panel) and to vitronectin (Vn, lower panel). The specific activity of phosphotyrosine content in PYK2 was determined by normalizing to the protein level. PYK2

tyrosine phosphorylation is expressed relative to control (in the absence of blocking antibodies), which is arbitrarily set at 1.0. Bars represent values from three separate experiments.

FIGURE 7 demonstrates that clustering of β_2-integrin induces tyrosine phosphorylation of PYK2 in macrophages. IC-21 cells were incubated with either anti-β_2 integrin mAb M18/2 or anti-β_1 integin mAb 9EG7. Then, cells were treated with goat F(ab')$_2$ anti-rat IgG (50 μg/ml) for the indicated times. Cells were lysed and subjected to immunoprecipitation and immunoblot using anti-phosphotyrosine mAb 4G10 and anti-PYK2 antibodies as described.

Figure 8 is the cDNA sequence of mouse PYK2 and the deduced protein sequence. Intron sequences are in lower case letters. The exon sequence is capitalized. The boxed sequence of the deduced protein indicates the kinase domain. The circled prolines of the deduced protein indicate the proline rich domain.

As used throughout this specification and claims, the following abbreviations are used:

ECM is extracellular matrix;
COL I is collagen type I;
COL IV is collagen type VI;
Fb is fibrinogen;
Fn is fibronectin;
Ln is laminin;
Poly-L-Lys is poly-L-lysine;
Vn is vitronectin;
FAK is focal adhesion kinase;
PYK2 is protein tyrosine kinase 2;
CAKβ is cell adhesion kinase β;
RAFTK is related adhesion focal tyrosine kinase.

One of the key features of this invention is the elucidation of the previously unappreciated biological functions of PYK2. In accordance with this invention it has been determined that PYK2 is

the kinase which is primarily responsible for podosome formation in monocyte-macrophages.

Further, in accordance with this invention it has been demonstrate that cell adhesion-dependent PYK2 activation occurs in macrophages. Moreover, PYK2 is specifically localized in macrophage podosomes and its activity is regulated by selective interaction with the integrin $\alpha_M\beta_2$.

In recognition that PYK2 is an appropriate target for compounds intended as PYK2 inhibitors or promotors, one aspect of this invention are assays to determine if a candidate molecule can effect PYK2 activity.

The assays of this invention which asses a compound's ability to modulate PYK2 activity may be cell-based or may use PYK2 which is no longer in intact cells. For cell based assay systems, virtually any cell which expresses PYK2 (either naturally or recombinantly) may be used. Such cells and cell lines which naturally express PYK2 are known to those in the art and include: macrophages, osteoclasts, phagocytes, and particularly immortalized mouse peritoneal IC-21 macrophage cell line.

If a recombinant cell expressing PYK2 is to be used, then any host cell which can be transformed and is capable of transcribing and translating nucleic acids encoding PYK2 may be used. Convenient host cells, including mammalian, yeast, and bacterial cells are known to those in the art. The sequence of mouse PYK2 is given in Figure 8 (SEQ ID NO:6).

The assays of this invention may be adaptations of any known assay. For example, one embodiment of this invention is a binding assay wherein either the compound to be assayed or PYK2 is labeled. Labels may be chemiluminescent, radioactive, flourescent or any labels routinely used in the art. In these assays, the compound and the PYK2 are contacted, and incubated for at least a sufficient time for binding to occur. The bound compound-PYK2 can then be separated from unbound compound and unbound PYK2 and the amount of bound entity can be determined by measuring the label.

Another assay in accordance with this invention is an *in vitro* kinase assay. In this assay, the activity of PYK2 activity is determined by measuring the ability of PYK2 to incorporate a labeled phosphate into a substrate. In preferred embodiments, the phosphate is radiolabeled, and its incorporation into poly-Glutamine or poly-Tyrosine by PYK2 is measured. A potential inhibitor or activating compound is added, and the incorporation rate is compared to the rate of incorporation in the absence of the compound. One embodiment of this assay is exemplified in Example 8.

Yet another assay in accordance with this invention measures the ability of PYK2 to phosphorylate itself at tyrosine residue 402. This assay is generally performed using conditions similar to those for the *in vitro* kinase assay, except that no substrate is required to be present. The incorporation of labeled phosphate into PYK2 is monitored in the presence and absence of the putative inhibitor. In preferred embodiments, the phosphate is radiolabeled and its incorporation into PYK2 is monitored by SDS-PAGE followed by X-ray radiography. The amount of auto-phosphoylation of PYK2 generally reflects the activation state of PYK2. Thus, a compound which inhibits autophosphoylation would be a compound which inactivates the kinase.

Still another assay in accordance with this invention is an assay which measures the effect (either inhibitory or stimulatory) a candidate compound has an podosome formation in a cell. The cells which may be use in this assay include any cell of interest which is known to form podosomes. If the candidate compound has potential use in osteoporosis, the preferred cell is an osteoclast or osteoclast-like cell. Podosomes are treated by methods known in the art so that they can be visualized, for example, by immunofluorescence. Any inhibitory effect of the candidate compound can then be visually assessed.

The above assays, which can identify and characterize a compound's ability to inhibit (or activate) PYK2 activity can therefore be used for a variety of endpoints. Thus, since inhibition of PYK2 can lead to the inhibition of podosome formation, prevention of monocyte

adhesion to a substrate, inhibition of osteoclast mobility and inhibition of extracellular matrix degradation, the above assays are useful for identification of compounds with these utilities.

Adhesion-dependent regulation of PYK2 activity was primarily examined in the immortalized mouse peritoneal IC-21 macrophage cell line. IC-21 shares many characteristics with normal (i.e., non-immortalized macrophages) peritoneal macrophages, including the ability to phagocytize, to secrete lysosomal enzymes, to function as effector cells in antibody-dependent cellular cytoxicity, and to respond to chemoattractants. IC-21 cells possess macrophage-specific antigens, Fc and C3 receptors.

Tyrosine phosphorylation of PYK2 is reduced in non-adherent IC-21 macrophages, while cell attachment and spreading on ECM increased PYK2 tyrosine phosphorylation and kinase activity in a time- and substrate-dependent manner. Activation of PYK2 appears to correlate with cell spreading, since macrophages attach to, but spread only slowly on poly-L-lysine, as compared to fibronectin. PYK2 tyrosine phosphorylation proceeded slowly as well.

Adhesion-induced PYK2 tyrosine phosphorylation and kinase activation suggests the involvement of integrins in the cell attachment and spreading process. Indeed, clustering of β_2 integrins, but not β_1 integrins, with the respective antibodies induce PYK2 tyrosine phosphorylation. Moreover, blocking antibodies to the integrin subunits α_M (M1/70) and β_2 (M18/2) which inhibit cell attachment and spreading on fibrinogen reduced PYK2 tyrosine phosphorylation on this substrate. Interestingly, when cells are seeded on fibronectin, blocking antibodies to $\alpha_M\beta_2$ inhibit PYK2 tyrosine phosphorylation, but not antibodies to the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ fibronectin receptors. Although $\alpha_4\beta_1$ and $\alpha_5\beta_1$ are the principal fibronectin receptors in IC-21 macrophages as well, blocking antibodies to either α_4 or α_5 did not substantially inhibit cell adhesion to fibronectin in this study.

Adhesion to vitronectin also induced PYK2 tyrosine phosphorylation, but flow cytometry analysis indicated low

expression of α_V-associated receptors and blocking antibodies to both $\beta 1$ or $\beta 2$ integrins had no effect on vitronectin-induced PYK2 phosphorylation.

After a period of time in the circulation, peripheral blood monocytes migrate to various tissues, where they are known to undergo final differentiation into macrophages. Monocyte migration involves multiple interactions with the endothelial lining, diapedesis between endothelial cells and crossing of the ECM. Podosomes or "rosette" adhesions have been detected in many transformed cells, but they are most abundant in spreading macrophages and osteoclasts. Interestingly, cells which express podosomes have an "invasive" phenotype, they are highly motile and secrete proteases. Podosomes are dynamic structures; they apparently assemble and disassemble within a few minutes. Podosomes have therefore been implicated in the regulation of rapid migration and in local degradation of the ECM. Inhibition of PYK2 activity thus results in reduced motility and decreased matrix degradation in macrophages. Therefore, the podosome-associated PYK2 is a potential crucial intermediate in adhesion-dependent differentiation and activation of macrophages.

PYK2 is highly expressed in macrophages and rapidly tyrosine phosphorylated upon cell attachment to specific ECM. This cell adhesion-dependent PYK2 phosphorylation is mediated, in part, by the ligation of integrin $\alpha M\beta 2$. In addition, PYK2 co-localizes with $\alpha M\beta 2$ to podosomes in macrophages. The localization of PYK2 implicates its function in the formation of podosomes and in the regulation of migration and matrix degradation of monocytic cells. To reach the above conclusions regarding PYK2 function, the following investigations were made.

A. Cloning and Expression of Mouse PYK2 and FAK

Since focal adhesion kinase (FAK) expression was unable to be detected in a number of macrophage cell lines and in bone marrow-derived osteoclasts, it was hypothesized that another

cell adhesion-dependent kinase, homologous to FAK, may assume its function in these cells. To evaluate PYK2 as a possible adhesion-dependent kinase in macrophages, specific probes were generated for PYK2 and FAK which were used to examine the expression of PYK2 and FAK in mouse tissues. As previously reported, PYK2 is highly expressed in brain and spleen, and at lower levels in kidney, lung and liver (Fig. 1, upper panel), and has a more restricted tissue distribution than FAK (Fig.1, lower panel).

Using the PYK2 probe, the full length cDNA from a mouse spleen cDNA library was cloned. The deduced amino acid sequence of the full length clone was found to be identical to the recently published amino acid sequence of the mouse RAFTK (Avraham, et al., 1995 *supra*). In addition, full length FAK from a mouse osteoblastic MB1.8 cell line (Wesolowski, et al., 1995 *Exp. Cell Res.* 219:679-686), and its sequence was the same as that published (Hanks, et al., 1992 *Proc. Natl. Acad. Sci. USA* 89:8487-8491). PYK2 and FAK cDNAs were subsequently transfected into human embryonic kidney (HEK) 293 cells. Cell lines which permanently express either PYK2 or FAK were established and the expression levels of the exogeneously expressed mouse kinases were assessed by northern analysis.

B. Characterization of Polyclonal Anti-PYK2 Antibodies

To study the function of PYK2 in macrophages, anti-PYK2 antibodies were developed against the C-terminal domain of mouse PYK2 as described in the Examples, and were affinity purified using the recombinant peptide. To characterize the polyclonal anti-PYK2 antibodies, or FAK was immunoprecipitated from the parental HEK 293 cells and from the transfected cell lines using either polyclonal anti-PYK2 antisera or monoclonal anti-FAK antibody (mAb 2A7), followed by immunoblotting with the respective antibodies. The parental HEK 293 cells express endogeneous FAK, but not PYK2 (Fig. 2). Anti-PYK2 antisera recognize a 110 kDa protein in HEK 293 cells transfected with full length mouse PYK2 cDNA (Fig. 2, lane 3), but not the transfected mouse FAK (Fig. 2, lane

2) or the endogenously expressed human FAK (Fig. 2, lane 1-3). In addition, PYK2 protein was not detected in NIH-3T3 cells and transformation of this fibroblastic cell line by either v-ras or v-src did not induce PYK2 (Fig. 2, lane 4-6).

C. PYK2 is expressed in Murine Macrophages

Using the same polyclonal anti-PYK2 antibodies, PYK2 expression was observed in isolated primary cultures of murine peritoneal macrophages (Fig. 3, lane 1) and in a number of mouse monocyte-macrophage cell lines (Fig. 3, lanes 2-5) as well as in bone marrow derived macrophages and osteoclast-like cells (Fig. 3, lanes 7-8). Previous studies suggested that monocytic cells do not express FAK. In this study, the lack of FAK expression in the peritoneal and bone marrow derived macrophages (Fig. 3, lanes 3 and 7) and in the bone marrow derived osteoclast-like cells was confirmed (Fig. 3, lane 8). FAK was not detected in the monocyte-macrophage cell lines, WEHI-3 and P388D1 (Fig. 3, lanes 4 and 5), and in the peritoneal macrophage IC-21 line (Fig. 3, lane 2). FAK was detected in the mouse bone marrow derived macrophage RAW264.7 cell line (Fig. 3, lane 3).

PYK2 which is highly expressed in all murine macrophages (Fig. 3, upper panel), appears to present as two forms, differing slightly in molecular weight. The peritoneal macrophage IC21 cells express both forms equally, while WEHI-3 and the bone marrow derived osteoclast-like cells express mainly the higher molecular weight PYK2. RAW264.7, P388D1, the primary peritoneal macrophages and the bone marrow derived macrophages express predominantly the lower molecular weight form of PYK2. PYK2 tyrosine was rapidly dephosphorylated upon trypsinization, but both PYK2 forms are still detected by anti-PYK2 antibodies, as marked by the arrows.

D. Substrate-dependent Cell Adhesion Induces Tyrosine Phosphorylation of PYK2 in Macrophages

It has been reported that the adhesion of rat fibroblast 3Y1 cells to fibronectin failed to induce tyrosine phosphorylation of PYK2 (Sasaki, et al., 1995 *J. Biol. Chem.* 270:21206-21219), however, attachment of CMK cells to fibronectin stimulated PYK2 tyrosine phosphorylation and kinase activity (Li, et al., 1996 *Blood* 88:417-428). In accordance with this invention it was found that in IC-21 macrophages in suspension, PYK2 is dephosphorylated (Fig. 4, lane 1). However, when the cells are replated on plastic tissue culture dishes in the presence of serum, PYK2 is rapidly tyrosine phosphorylated by immunoblotted with mAb 4G10 as the cells attach and spread (Fig. 4, lane 2). Similar adhesion-induced tyrosine phosphorylation of PYK2 was also observed in the other monocyte-macrophage cell lines and in primary macrophages. Substantial tyrosine phosphorylation of PYK2 in IC-21 cells seeded on ECM-coated polystyrene dishes in the absence of serum for 20 min at 37°C is induced only by specific ECM components: fibronectin, vitronectin or fibrinogen (Fig. 4, lanes 3-9). Much lower levels of PYK2 phosphorylation are also detected when cells are plated on polylysine, collagen type I, collagen type IV, or laminin. Previous reports on laminin receptors suggest that in macrophages they are normally in a low affinity state and require activation for adhesion to laminin-coated surfaces.

Adhesion-dependent PYK2 tyrosine phosphorylation is a rapid response. Upon attachment of IC-21 macrophages to either fibronectin, vitronectin or fibrinogen, an increase in PYK2 tyrosine phosphorylation is detected within 1 minute and peaks around 20 minutes after plating (Fig. 5A and 5B). In addition, the increase in PYK2 tyrosine phosphorylation upon attachment to fibronectin is associated with a concomittant increase in PYK2 intrinsic kinase activity (Fig. 5C). Attachment to poly-L-lysine caused a much slower increase in PYK2 tyrosine phosphorylation (Fig. 5B), which paralleled slower spreading of IC-21 cells on poly-L-lysine coated surfaces. Interestingly, PYK2 is highly phosphorylated for as long as the cells are allowed to attach and spread on the ECM coated dishes.

No decline in PYK2 tyrosine phosphorylation or kinase activity was observed for up to 1 hour and for as long as 4 hours.

E. Induction of PYK2 Tyrosine phosphorylation is Mediated by Integrin $\alpha_M\beta_2$

Since the $\beta 1$ and $\beta 2$ integrins are primarily responsible for the adherence of macrophages to other cells and to ECM components such as fibronectin and fibrinogen and α_V integrins mediate binding to vitronectin the role of these integrins in the adhesion-induced tyrosine phosphorylation of PYK2 was examined. The surface expression of integrins present in IC-21 macrophages was determined by flow cytometry. As shown in Figure 6A, the predominant integrins are $\alpha 4\beta 1$ and $\alpha_M\beta 2$. Lower levels of integrins $\alpha 5\beta 1$ and $\alpha_L\beta 2$ were also detected. However, integrins $\alpha_X\beta 2$ and the α_V-associated integrins were not detected in this cell line, using mAb HL3 and mAb H9.2B8, respectively.

To examine the role of specific integrins in PYK2 tyrosine phosphorylation, IC-21 macrophages were incubated with blocking antibodies to the $\beta 2$-associated integrin subunits: anti-integrin α_L antibody (M17/4), anti- integrin α_M antibody (M1/70) or anti- integrin $\beta 2$ antibody (M18/2). In addition, blocking antibodies to the integrin subunit $\alpha 4$ (R1-2), or $\alpha 5$ (MFR5) and to the integrin subunit $\beta 1$ (9EG7) were also used. As shown in Figures 6B and 6C, PYK2 tyrosine phosphorylation is specifically inhibited by anti-α_M and anti-$\beta 2$ antibodies when IC-21 cells are seeded on fibrinogen or fibronectin. Surprisingly, when the cells adhere to fibronectin-coated plates, antibodies to the integrin subunit $\alpha 4$, $\alpha 5$ or $\beta 1$ fail to block the increase in PYK2 tyrosine phosphorylation. On vitronectin, antibodies to the $\beta 2$-associated integrins or to the $\beta 1$-associated integrins do not prevent the adhesion-mediated PYK2 phosphorylation in IC-21 cells (Fig. 6C). Although, the expression of the α_V-associated integrins could not be demonstrated in this study using flow cytometry, the possibility of low expression of α_V integrins sufficient to mediate macrophage attachment to vitronectin cannot be

ruled out. These findings suggest that the integrin $\alpha_M\beta_2$ is the predominant receptor which mediates IC-21 macrophage attachment to fibrinogen, and regulates cell attachment to fibronectin. IC-21 cells express significant levels of integrin $\alpha_4\beta_1$ and detectable levels of $\alpha_L\beta_2$, and $\alpha_5\beta_1$ as shown by flow cytometry (Fig. 6A), however these receptors do not appear to play a significant role in regulating PYK2 phosphorylation during the initial phase within 20 minutes of cell adhesion to fibrinogen and fibronectin.

The role of integrin $\alpha_M\beta_2$ in mediating PYK2 tyrosine phosphorylation was further supported by integrin ligation. IC-21 macrophages were incubated either with anti-integrin β_2 antibodies (M18/2) or anti-integrin β_1 antibodies (9EG7), and clustering effects were enhanced by incubation with secondary antibodies. PYK2 phosphotyrosine levels were determined after immunoprecipitation by immunoblotting. As shown in Figure 7, ligation of the β_2 integrin subunit (lane 1-5) increases PYK2 tyrosine phosphorylation, while ligation of the β_1 integrin subunit (lane 6) has no effect. Similar to cell attachment, ligation of the β_2 integrin causes a very rapid (within 5 minutes) increase in PYK2 tyrosine phosphorylation, which remains elevated at least up to 30 minutes at 37°C.

F. PYK2 Localizes to Podosomes in Macrophages

The localization of PYK2 in IC-21 cells and in primary bone marrow-derived and peritoneal macrophages was examined. Cells were allowed to adhere on fibronectin-coated glass coverslips in the absence of serum. The same findings as those described below were obtained using vitronectin- or fibrinogen-coated coverslips. After 20 hours at 37°C, all cells are spread and display a typical fan-like shape of migrating macrophages. Immunofluorescent staining of both IC-21 peritoneal macrophages and primary macrophages with affinity purified anti-PYK2 antibodies visualize PYK2 either in the perinuclear region or in structures resembling podosomes. Depending on the state of cell migration, the podosome-associated PYK2 was found in the periphery of the ruffled-leading edge of motile cells or organized in extensive arrays, mainly underneath the migrating cell bodies and occasionally under the nucleus. Similar to focal adhesion kinase, podosome associated PYK2 was always found to cluster with proteins highly tyrosine phosphorylated. This is evidence of a role for PYK2 in the assembly and/or disassembly of podosomes in macrophages.

G. Co-localization of PYK2 and Cytoskeletal Proteins

Because PYK2 localizes to podosomes, it may play an important role in anchoring actin filaments. When IC-21 macrophages were co-stained with anti-PYK2 antibodies and phalloidin, PYK2 was detected extensively in the perinuclear regions of some cells, where it was never found to associate with F-actin. However, podosome associated PYK2 (which appeared as dot-like structures) was readily demonstrated to cluster with aggregrates of F-actin. Similarly, podosome-associated PYK2 was found either at the leading edge or under the lamellipodia and the migrating cell body. Immunostaining of α-actinin also revealed its co-localization with PYK2 in podosome structure.

In accordance with this invention, PYK2 was found to be organized as rings in podosome adhesion contacts in macrophages.

This indicates that PYK2 is closely associated with a number of cytoskeletal proteins, including vinculin, talin and paxillin, which were previously identified to form ring-like structures surrounding the actin core in podosomes. To further confirm the subcellular distribution of PYK2 in macrophages, IC-21 cells were double stained with anti-PYK2 antibodies and monoclonal antibodies to vinculin, talin or paxillin. PYK2 was again shown to concentrate in podosomes as well as in the perinuclear region. Co-localization of PYK2 with vinculin with talin and with paxillin in the ring-like structure was demonstrated.

H. Co-localization of PYK2 and the Integrin $\alpha_M\beta_2$ in Macrophages

The β2 integrin was previously detected as a diffusion corona of staining around the podosome adhesion contacts in the monocyte-macrophage cell lineage. Since the present functional data suggested the involvement of the integrin $\alpha_M\beta_2$ in the activation of PYK2 in peritoneal macrophage IC-21 cells, the association of this kinase with the integrin $\alpha_M\beta_2$ was examined by double staining IC-21 cells plated on fibronectin-coated glass coverslips, with both anti-PYK2 antibodies and the anti-α_M integrin subunit or the anti-β_2 integrin subunit. It was found the two structures to co-localize. Immunostaining of IC-21 cells with anti-α_L integrin, anti-α_4 integrin, anti-α_5 integrin and anti-β_1 integrin was also performed. These integrins appeared to be diffusely located in the apical surface of IC-21 macrophages. No co-localization of the β_1-associated integrins with PYK2 was observed in IC-21 macrophages.

The following non-limiting Examples are presented to better illustrate this invention.

EXAMPLE 1

Antibodies

Monoclonal anti-FAK antibody 2A7 was purchased from Upstate Biotech. (UBI, Lake Placid, NY). The following rat anti-mouse β_2 associated integrins were purified from hybridoma supernatants obtained from the American Type Culture Collection (ATCC, Rockville, MD): mAb M17/4 (anti-a_L), mAb M1/70 (anti-α_M), and mAb M18/2 (anti-β_2). Rat anti-mouse α_4 integrin mAb R1-2 was a gift from Dr. Irving L. Weissman, Stanford University. Monoclonal antibodies to integrin subunits α_5 (mAb MFR5), α_v (mAb H9.2B8), α_x (mAb HL3) and β_1 (mAb 9EG7) were purchased from Pharmingen, San Diego, CA. Antibodies to phosphotyrosine (mAb py20) and paxillin (mAb 349) were from Transduction Labs. (Lexington, KY). Antibodies to vincullin (mAb VIN-11-5) and to talin (mAb 8d4) were from Sigma (St. Louis, MO). F(ab)'2 anti-rat IgG, FITC-conjugated goat anti-mouse IgG and TRITC- conjugated donkey anti-rabbit IgG were purchased from Jackson Labs (West Grove, PA). FITC-conjugated goat anti-rat IgG and FITC-conjugated goat anti-hamster IgG were purchased from Boehringer Mannheim Co.,. (Indianapolis, IN). All horseradish peroxidase (HRP) conjugated secondary antibodies were purchased from Amersham (Arlington Heights, IL), except the direct HRP-conjugated anti-phosphotyrosine mAb 4G10 was from UBI. All secondary antibodies coupled to Sepharose were from Organon Teknika (Durham, NC).

EXAMPLE 2

Cell Culture

All monocyte/macrophage cell lines, IC-21 (ATCC, TIB-186), P388D1 (ATCC, TIB-63), RAW264.7 (ATCC, TIB-71), WEHI-3 (ATCC, TIB-68), were obtained from the American Type Culture Collection (Rockville, MD). Murine peritoneal macrophages were prepared as described (Mercurio, et al., 1984, *J. Exp. Med.* 160:1114-1125, which is hereby incorporated by reference. Briefly, macrophages were induced by thioglycolate injection into the peritoneal cavities of adult BALB/c mice. After 4 days, cells were collected, washed and cultured in RPMI 1640 medium containing 10% FBS. After 3 hours at 37°C, the cultures were washed extensively to remove non-adherent cells and cultured overnight before samples were prepared for immunoprecipitation. Bone marrow derived macrophages were prepared as described (Li and Chen, 1995, *J. Leuk. Biol.* 57:484-490, which is hereby incorporated by reference). Non adherent cells were cultured in RPMI completed medium in the presence of human macrophage colony-stimulating factor (MCS-F, 250 units/ml, Genetics Institute, Cambridge, MA). Differentiated macrophages were prepared for immunoprecipitation after 5 days in culture. Bone marrow derived osteoclast-like cells were prepared as described (Wesolowski, et al., 1995 *Exp. Cell Res.* 219:679-686, which is hereby incorporated by reference). After the collagenase-dispase treatment, mononucleated tartrate resistant phosphatase positive cells were released from the tissue culture plate using 30 nM echistatin. Freshly isolated osteoclast-like cells were immediately solubilized in immunoprecipitation buffer.

EXAMPLE 3

cDNA Cloning and Expression of mouse PYK2

Specific probes for mouse PYK2 and FAK were initially generated based on the non-homologous region between the proteins, which is adjacent to the C-terminal of the kinase domain. Using polymerase chain reaction (PCR), a specific probe for PYK2 (570bp) was generated using the 5'-primer (AGTGA CATTT ATCAG ATGGA G) (SEQ.ID.NO. 1) and the 3'-primer (GAATG GACTG TGCAC CGAGC C) (SEQ.ID.NO.2), with cDNAs of mouse bone marrow derived osteoclast-like cells as template (Wesolowski, et al., 1995, *supra*). Similarly, a specific probe for FAK (700bp) was generated using the following primers: 5'- (CAGCA CACAA TCCTG GAGGA G) (SEQ. ID.NO.3) and 3'- (GCTGA AGCTT GACAC CCTCA T) (SEQ.ID.NO.4) with cDNAs of mouse osteoblastic MB1.8 cells as template (Wesolowski, et al., 1995, *supra*). These probes were confirmed by sequencing analysis. PYK2 cDNA fragments were cloned from a mouse spleen ZAP II cDNA library (Stratagene, La Jolla, CA) using the specific PYK2 probe. Full length PYK2 cDNA were constructed by ligation of two overlapping clones at the VspI site. The amino acid sequence of the isolated PYK2 cDNA clone was identical to the previously published mouse RAFTK sequence (Avraham, et al., 1995, *J. Biol. Chem.* 270: 27742-27751.) . Full length FAK cDNA was generated by PCR according to the published sequence (Hanks, et al., 1992 *Proc. Natl. Acad. Sci. USA*. 89:8487-8491, which is hereby incorporated by reference). Both PYK2 and FAK cDNAs were subcloned into pCDNA3 plasmid (InVitrogen, San Diego, CA) and transfected into human embryonic kidney (HEK) 293 cells (ATCC, Rockland, MD) by electroporation at 200V, 960 μF using a GenePulser (Biorad Labs, Richmond, CA). HEK 293 cells was subsequently subjected to G418 selection (800 μg/ml, Gibco BRL) and clones were picked after 3 weeks in selection medium.

Expression of PYK2 and FAK in HEK293 cells were confirmed by northern analysis using the respective probes and by western blot analysis using either polyclonal anti-PYK2 antibodies or

mAb 2A7 anti-FAK antibody. Mouse multiple tissue northern blot was purchased from Clonetech (Palo Alto, CA) and hybridization of the northern blot using probes specific for PYK2, FAK and glyceraldehde 3-phosphate dehydrogenase (GAPDH) were performed as described previously (Wesolowski, et al., 1995, *supra*).

EXAMPLE 4

Production and Affinity Purification of Polyclonal Antibodies to Mouse PYK2

The PYK2 C-terminal domain (from methionine residue 685 to end) was amplified by PCR using the mouse PYK2 as template. Amplified product was cloned into pGEX-4T plasmid (Pharmacia Biotech., Piscataway, NJ) and transformed in *E. coli* XL1-Blue (Stratagene). Expression of GST-PYK2 C-terminal fragment was induced using 0.5 mM IPTG, purified and cleaved from GST with thrombin, essentially according to the instructions of the manufacturer (Pharmacia). The purified C-terminal fragment of mouse PYK2 was used to immunize two rabbits (Research Genetics, Huntsville, AL) and the titers of both antisera were initially determined by ELISA using the recombinant C-terminal fragment of PYK2. Specificity of the immune sera was subsequently determined by western blot by comparison to the preimmune sera. Polyclonal antibodies were then affinity purified by passing the combined fractions of both antisera through an affinity column, which was constructed using the same purified antigen cross linked to CNBr-activated Sepharose 4B according to the instructions of the manufacturer (Pharmacia). The antibodies were eluted from the column using 0.2 M Glycine, pH 2.5 and 1mM EGTA and the eluted

fraction was then dialyzed against PBS containning 0.02% azide. Anti-PYK2 antibodies were stored at -70°C at a concentration of 0.5mg/ml.

EXAMPLE 5

Cell Attachment to ECM and Inhibition by Anti-Integrin Antibodies

Polystyrene dishes (35 mm, Becton Dickinson, Lincoln Park, NJ) were coated overnight at 4°C with either 100 µg/ml polylysine (Sigma), or 25 µg/ml human fibronectin (NY Blood Center, New York, NY), or 10 µg/ml human vitronectin, or 50 µg/ml human fibrinogen, or 25 µg/ml mouse laminin (Gibco BRL), or 25 µg/ml collagen type I or collagen type IV (Collaborative Biomed., Bedford, MA). Plates were blocked with blocking buffer containing casein (Pierce, Rockford, IL) for 1h at room temperature, rinsed with PBS prior to addition of cell suspensions. Cells were lifted using Trypsin-EDTA (5 min, 37°C) and washed 3 times with serum free RPMI medium containing soybean trypsin inhibitors (SBTI, 0.5 mg/ml, Sigma). Cells in suspension (2 X 10^6 cells per ml) were allowed to attach to ECM-coated plates at 37°C for 1 to 60 min as indicated. Cells were solubilized in RIPA buffer and prepared for immunoprecipitation.

Inhibition of cell attachment to ECM by blocking anti-integrin antibodies was performed essentially as followed: IC-21 macrophages were lifted using trypsin-EDTA and washed with serum free RPMI media containing SBTI as described above. Cell suspensions (2 X 10^6 cells per ml) were incubated with 25 µg of one of the following anti-mouse integrin subunit antibodies: mAb M17/4 (anti-α_L), mAb M1/70 (anti-α_M), mAb M18/2 (anti-β_2), mAb R1-2 (anti-α_4), mAb MFR5 (anti-α_5) or mAb 9EG7 (anti-β_1). Prior to inhibition of cell attachment, all antibodies were washed and concentrated (1mg/ml) on a Centricon-30 concentrator (Amicon, Beverly MA) in the presence of PBS and 0.1% BSA. After incubation with antibodies for 20 min, cells were allowed to attach to ECM -

coated plates for an additional 20 min at 37°C, prior to preparation for immunoprecipitation using anti-PKY2 antibodies (see below).

EXAMPLE 6

Integrin Clustering

Antibody-induced clustering in the peritoneal macrophage IC-21 cell line was performed as previously reported (Greenberg, et al., 1994 *J. Biol. Chem.* 269:3897-3902, which is hereby incorporated by reference). After trypsinization and washing as described above, cell suspensions (1 X 10^6 cells per ml) were incubated with mAb M18/2 or mAb 9EG7 (25 μg/ml) at 4°C for 30 min. Cells were washed with ice-cold serum free medium (2X) containing 100 μM sodium vanadate and resuspended in medium containing 50 μg/ml of goat F(ab)'2 anti-rat IgG and shifted into 37°C incubation for the indicated times. Cells were lyzed in RIPA buffer and subjected to immunoprecipitation and immunoblotting as described below.

EXAMPLE 7

Immunoprecipitation of PYK2 and FAK

To analyze the expression levels of PYK2 and FAK in various cell lines, total cell lysates were prepared by the addition of 1 ml ice cold RIPA buffer containing 1 mM sodium vanadate, 50 mM NaF and a cocktail of protease inhibitors containing 2mM PMSF, 20 μg/ml aprotinin, 10 μg/ml leupeptin (Boehringer Mannheim, Indianapolis, IN) and incubated for another 20 min for complete solubilization. After centrifugation, total protein concentration of the clarified lysates was determined. Typically, 250 μg of cell lysates were subjected to immunoprecipitation using either anti-PYK2 antibodies (1μg) or mAb 2A7 anti-FAK antibody (4 μg). Immunoprecipitation was carried out for at least 4 hrs at 4°C,

followed by addition of anti-rabbit IgG or anti-mouse IgG coupled to Sepharose (Organon Teknika). To study the phosphotyrosine content of PYK2 in IC-21 cells in response to cell adhesion, the attachment assay described above was stopped by addition of an equal volume of 2X ice cold RIPA buffer, and cell lysates were prepared for immunoprecipitation using 2 μg of anti-PYK2 antibodies. After SDS-PAGE and transfer to nitrocellulose membranes (Novex, San Diego, CA), phosphotyrosine was detected by immunoblotting with HRP-conjugated anti-phosphotyrosine mAb 4G10 or with anti-PYK2 polyclonal antibodies, followed by HRP-conjugated anti-rabbit IgG. Blots were developed by enhanced chemiluminescence (ECL, Amersham). ECL signals were determined using an LKB ultroscan XL laser densitometer (LKB, Bromma, Sweden) and the specific activity of tyrosine phosphorylated PYK2 was calculated by comparing the estimated phosphotyrosine contents to protein levels of PYK2. Relative specific activity of phosphorylated PYK2 was normally determined from triplicated experiments.

EXAMPLE 8

In vitro Kinase Assay

After cell attachment to ECM, IC-21 cells were solubilized in TNE lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1mM EDTA, 10% glycerol, 50 mM NaF, 1 mM sodium vanadate and protease inhibitors as described above. PYK2 was immunoprecipitated from the clarified lysates, half of the sample was subjected to immunoblotting with anti-PYK2 antibodies, as described above, and the other half was washed 2 times with the same lysis buffer, and with kinase assay buffer (1X) containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MnCl_2$ and 1 mM dithiothreitol. After removal of the wash buffer, 50 μl of kinase assay buffer containing 5 μCi [γ-^{32}P] ATP (3000Ci/mmol, Amersham), 10 μM ATP, 0.1% BSA and 100 μg of poly (Glu,Tyr) (molar ratio 4:1; Sigma) was added to the beads and incubated for 10 min at 30°C (Howell and Cooper, 1995 *Mol. Cell. Biol.* 14:5402-5411). The reaction

mixtures (25 µl) were added to 25 µl of 30% trichloroacetic acid (TCA) and 0.1 M sodium pyrophosphate, followed by incubation at 4°C for 15 min. The precipitated proteins were transferred to a Multiscreen-FC filter plate (Millipore, Marlborough, MA), washed with ice cold 15% TCA (3X), allowed to dry and incorporation of ^{32}P into the substrate was counted on a Packard top count microplate scintillation counter (Packard, Meriden, CT). Each assay was performed as triplicate. The specific activity was determined by comparing the radioactive counts with immunoblot signals.

EXAMPLE 9

Flow Cytometry

Surface expression of integrins was analyzed by single-color flow cytometry. After trypsin-EDTA treatment, cells were washed with completed RPMI media containing 10% FBS, twice with Dulbecco's phosphate buffer saline (DPBS) and resuspended in DPBS containing 1% BSA. Cells (2×10^4) were incubated with the anti-integrins mAbs (2µg), as described above, followed by incubation at 4°C for 30 min. The samples were washed once before addition of FITC- labeled goat anti-rat IgG or goat anti-hamster IgG (Boehringer Mannheim). After additional 30 min incubation at 4°C, cells were washed and resuspended in 300 µl of Flow Cytometric buffer (100 mM Hepes buffer, pH 7.5, 150 mM NaCl, 3 mM KCl and 1 mM $CaCl_2$) and analyzed by a FACSCalibur (Becton Dickinson, San Jose, CA).

EXAMPLE 10

Immunofluorescence Microscopy

Immunofluorescent labeling of podosomes in IC-21 cells was performed as followed: IC-21 cells were lifted by trypsin-EDTA for 5 min., washed in serum free media (2X), plated on Fn coated glass coverslips and left overnight at 4°C. Cells were washed in PBS (2X) and fixed for 10 min in 4% paraformaldehyde, 2% sucrose in

PBS. Cells were then permeabilized in 0.5% Triton, PBS for 5 min, followed by incubation for 1 hr in blocking buffer containing 10% normal goat serum, 1% BSA in PBS. All subsequent incubations with primary and secondary antibodies were performed in the same blocking buffer. PYK2 was visualized using the affinity purified polyclonal anti-mouse PYK2 antibodies, followed by TRITC- donkey anti-rabbit IgG. Actin was stained with 500 mU/ml FITC-phalloidin (Molecular Probes, Inc., Eugence, OR). Phosphotyrosine and paxillin were stained with mouse mAb py20 and mAb 349, respectively. Vinculin and talin were stained using mouse mAb VIN-11-5 and mAb 8d4, respectively. The mouse monoclonal antibodies were visualized using FITC- goat anti-mouse IgG. The integrin subunits α_L, α_M, α_4, α_5, β_1 and β_2 were immunostained using the following rat anti-mouse integrin antibodies: M17/4, M1/70, R1-2, MFR5, 9EG7 and M18/2, respectively, followed by FITC-conjugated goat anti-rat IgG. Immunofluorescent labelled cells were photographed through an 100X objective using a Zeiss Axiophot epifluorescence microscope.

Co-localization of PYK2 and Phosphotyrosine in Macrophage Podosomes

IC-21 cells were plated on fibronectin-coated glass coverslips in serum-free media. Migrating macrophages with typical fan-like shape were fixed and solubilized. Cells were co-stained for PYK2 using affinity purified anti-PYK2 polyclonal antibodies, followed by TRITC-donkey anti-rabbit IgG, and for phosphotyrosine using mAb py20, followed by FITC-goat anti-mouse IgG. PYK2 appeared as a ring structure in the adhesion contacts, organized in the cell leading edge or in extensive arrays of rosettes under the cell body. The phosphotyrosine appeared as dot-like structures, which predominantly co-localize with PYK2 in macrophages.

Podosome-Associated PYK2 co-localized with F-actin in Macrophages

IC-21 cells were co-stained with FITC-phalloidin and anti-PYK2 antibodies, followed by TRITC-donkey anti-rabbit IgG. A typical migrating macrophage with a typical fan-like shape or a macrophage with multiple adhesion contacts was chosen. PYK2 localized to perinuclear and dot-like structures at the leading edge or to extensive arrays of podosomes underneath the lamellaepodia. In the same cells, F-actin cores concentrated in podosomes. Co-localization of PYK2 and F-actin was detected in podosomes and tail regions of migrating macrophages while perinuclear PYK2 was not associated with actin filaments.

PYK2 Co-localizes with Vinculin, Talin and Paxillin in Podosomes of Macrophages

IC-21 cells were co-stained with anti-PYK2 antibodies and with anti-vinculin mAb VIN-11-5, with anti-talin mAb 8d4, and anti-paxillin mAb 349, followed by appropriate conjugated secondary antibodies. PYK2 localized in the perinuclear regions and in podosomes. Only podosome associated PYK2 was co-localized with vinculin, talin and paxillin, which all appear as ring-like structures.

Co-localization of PYK2 and the Integrin $\alpha_M\beta_2$ in Macrophages

IC-21 cells were plated on fibronectin-coated surface and stained with anti-PYK2 antibodies and with rat anti-mouse α_M mAb M1/70, rat anti-mouse β_2 mAb M18/2, followed by TRITC-donkey anti-rabbit IgG and FITC-goat anti-rat IgG.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: DUONG, LE T.
RODAN, GIDEON A.

(ii) TITLE OF THE INVENTION: IDENTIFICATION OF INHIBITORS OF PROTEIN TYROSINE KINASE 2

(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:
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(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAATGGACTG TGCACCGAGC C 21

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAGCACACAA TCCTGGAGGA G 21

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCTGAAGCTT GACACCCTCA T 21

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3981 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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TGCGCTCACC TGGCCCAGCC CGGAGCCCTG GCCCGAGTCC GCGCCTCGCC CGAGGGACTG 120
CAATGTGCCG GTCCTAGCTG CAGTCTGAGA GGATGTCCGG GGTGTCTGAG CCCTTGAGCC 180
GTGTAAAAGT GGGCACTTTA CGCCGGCCTG AGGGCCCCCC AGAGCCCATG GTGGTGGTAC 240
CAGTGGATGT GGAGAAGGAA GACGTGCGCA TCCTCAAGGT CTGCTTCTAC AGCAACAGCT 300
TCAACCCAGG GAAGAACTTC AAGCTTGTCA AATGCACAGT GCAGACAGAG ATCCAGGAGA 360
TCATCACCTC CATCCTCCTG AGTGGGCGAA TAGGGCCCAA CATCCAGCTG GCTGAATGCT 420
ATGGGCTGAG GCTGAAGCAC ATGAAGTCAG ACGAGATCCA CTGGCTGCAC CCACAGATGA 480
CCGTGGGCGA AGTGCAGGAC AAGTATGAAT GTCTACACGT GGAAGCTGAG TGGAGGTATG 540
ACCTTCAAAT CCGCTACTTG CCGGAAGACT TCATGGAGAG CCTGAAAGAA GACAGGACCA 600
CATTGCTGTA CTTTTATCAA CAGCTCCGGA ATGACTACAT GCAACGCTAC GCCAGCAAGG 660
TCAGTGAAGG CATGGCTCTG CAGCTGGGCT GTCTGGAGCT CAGGAGATTC TTCAAGGACA 720
TGCCCCACAA TGCACTGGAC AAAAAGTCCA ACTTTGAACT CCTGGAAAAA GAAGTCGGTC 780
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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1009 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Ser Phe Asn Pro Gly Lys Asn Phe Lys Leu Val Lys Cys Thr Val Gln
    50                  55                  60
Thr Glu Ile Gln Glu Ile Ile Thr Ser Ile Leu Leu Ser Gly Arg Ile
65                  70                  75                  80
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                85                  90                  95
Met Lys Ser Asp Glu Ile His Trp Leu His Pro Gln Met Thr Val Gly
            100                 105                 110
Glu Val Gln Asp Lys Tyr Glu Cys Leu His Val Glu Ala Glu Trp Arg
        115                 120                 125
Tyr Asp Leu Gln Ile Arg Tyr Leu Pro Glu Asp Phe Met Glu Ser Leu
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Lys Glu Asp Arg Thr Thr Leu Leu Tyr Phe Tyr Gln Gln Leu Arg Asn
145                 150                 155                 160
Asp Tyr Met Gln Arg Tyr Ala Ser Lys Val Ser Glu Gly Met Ala Leu
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Gln Leu Gly Cys Leu Glu Leu Arg Arg Phe Phe Lys Asp Met Pro His
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        195                 200                 205
Gly Leu Asp Leu Phe Phe Pro Lys Gln Met Gln Glu Asn Leu Lys Pro
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625                 630                 635                 640
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Pro Met Glu Tyr Pro Ser Pro Val Asn Ser Leu His Thr Pro Pro Leu
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    770                 775                 780
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785                 790                 795                 800
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Glu Asp Ser Gln Trp Leu Arg Arg Glu Glu Arg Cys Leu Asp Pro Met
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Val Tyr Met Asn Asp Lys Ser Pro Leu Thr Pro Glu Lys Glu Ala Gly
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Gln Ser Ile Gln Pro Thr Ala Asn Leu Asp Arg Thr Asp Asp Leu Val
865                 870                 875                 880
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                885                 890                 895
Lys Leu Gly Gln Leu Pro Pro Glu Asp Tyr Val Val Val Val Lys Asn
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Pro Ser Leu Pro Ala Ser Ser Arg Thr Glu Ile Glu Gly Thr Gln Lys
    930                 935                 940
Leu Leu Asn Lys Asp Leu Ala Glu Leu Ile Asn Lys Met Lys Leu Ala
945                 950                 955                 960
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            980                 985                 990
Ala Val Asp Gln Ala Lys Val Val Ala Asn Leu Ala His Pro Pro Ala
        995                1000                1005
Glu
  1
```

WHAT IS CLAIMED IS:

1. A method of identifying a compound which binds to and/or modulates the activity of Protein Tyrosine Kinase 2 (PYK2) comprising:

a) contacting the compound and PYK2; and

b) determining if binding has occurred.

2. A method according to Claim 1 further comprising the step of comparing activity of PYK2 which has bound to the compound to activity of PYK2 which is not bound to the compound.

3. A method according to Claim 1 wherein the PYK2 is present in intact cells.

4. A method according to Claim 1 wherein the PYK2 is not in an intact cell.

5. A method according to Claim 3 wherein the intact cell is a recombinant cell which expresses PYK2.

6. A method according to Claim 2 wherein the compound is labeled.

7. A method according to Claim 1 wherein the PYK2 is labeled.

8. A method according to Claim 2 wherein the activity of PYK2 is determined by measuring the ability of PYK2 to incorporate a labeled phosphate into a poly-glutamine or poly-tyrosine substrated.

9. A method according to Claim 8 wherein the labeled phosphate is radiolabeled.

10. A method according to Claim 2 wherein the activity of PYK2 is determined by measuring the ability of PYK2 to incorporate labeled phosphate into itself at tyrosine residue 402.

11. A method according to Claim 10 wherein the phosphate is radio-labeled.

12. A method according to Claim 12 wherein the intact cell forms podosomes in the absence of compound.

13. A method according to Claim 12, wherein step b) comprises measuring the effect the compound has on podosome formation in the cell.

14. A method of identifying a compound which prevents monocyte adhesion to a substrate by determining the ability of the compound to inhibit Protein Tyrosine Kinase 2 (PYK2) activity comprising:

a) contacting a compound with PYK2; and

b) determining if the compound inhibits PYK2 activity.

15. A method according to Claim 14 wherein step b) comprises a method selected from the group consisting of:

a) measuring the ability of the compound to inhibit the ability of PYK2 to incorporate phosphate into a poly-glutamine or poly-tyrosine substrate;

b) measuring the ability of the compound to inhibit the ability of PYK2 to incorporate phosphate into itself at tyrosine residue 402; and

c) measuring the ability of the compound to inhibit the formation of podosomes

16. A method of identifying a compound which inhibits osteoclast mobility by determining the compound's ability to inhibit Protein Tyrosine Kinase (PYK2) activity comprising:

a) contacting a compound with PYK2; and

b) determining if the compound inhibits PYK2 activity.

17. A method according to Claim 16 wherein step b) comprises a method selected from the group consisting of:

a) measuring the ability of the compound to inhibit the ability of PYK2 to incorporate phosphate into a poly-glutamine or poly-tyrosine substrate;

b) measuring the ability of the compound to inhibit the ability of PYK2 to incorporate phosphate into itself at tyrosine residue 402; and

c) measuring the ability of the compound to inhibit the formation of podosomes.

18. A method of identifying a compound which inhibits a monocytic cell from degrading an extracellular matrix by determining the compound's ability to inhibit Protein Tyrosine Kinase (PYK2) activity comprising:

a) contacting a compound with PYK2; and

b) determining if the compound inhibits PYK2 activity.

19. A method according to Claim 18 wherein step b) comprises a method selected from the group consisting of:

a) measuring the ability of the compound to inhibit the ability of PYK2 to incorporate phosphate into a poly-glutamine or poly-tyrosine substrate;

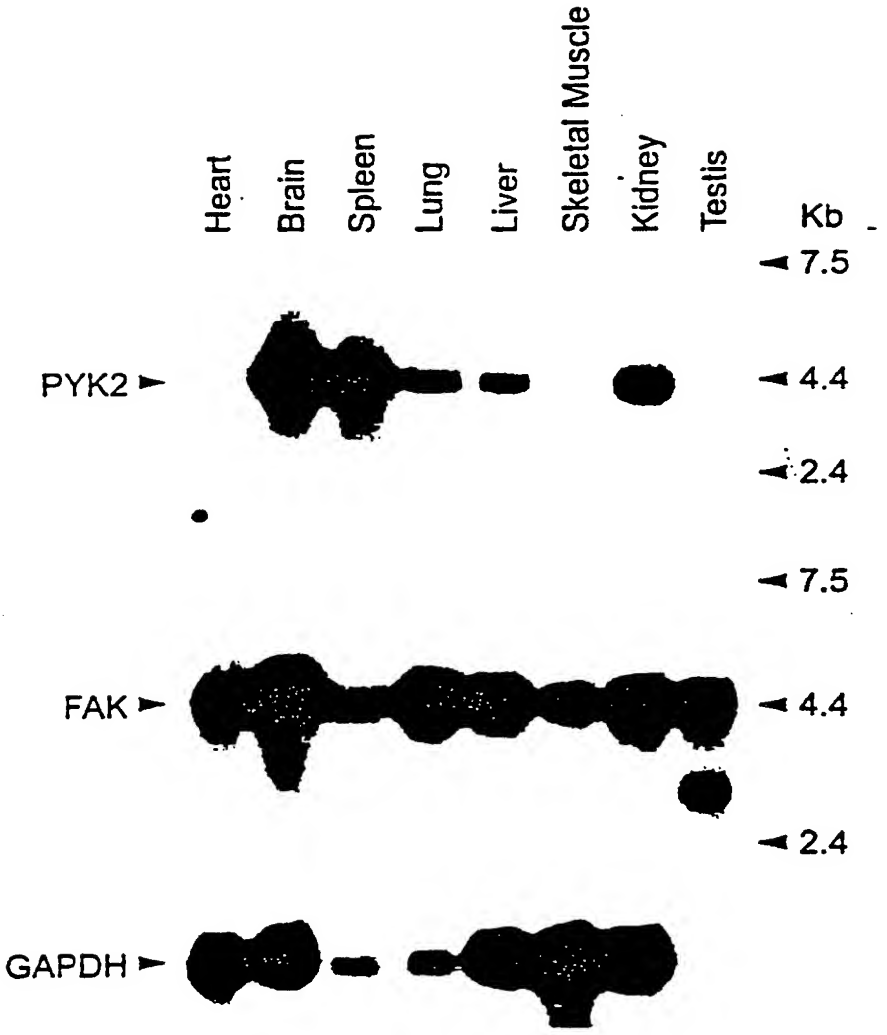
b) measuring the ability of the compound to inhibit the ability of PYK2 to incorporate phosphate into itself at tyrosine residue 402; and

c) measuring the ability of the compound to inhibit the formation of podosomes.

20. A compound identified according to the method of Claim 1.

21. A method of treating or preventing a disease state or condition in a mammal which is mediated by PYK2 comprising administering a compound according to Claim 20.

22. A method of treating or preventing osteoporosis or inflammation in a mammal comprising administering a compound according to Claim 20.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Brain
Spleen
Lung
Liver
Skeletal Muscle
Kidney
Testis
Kb
7.5
PYK2
4.4
2.4
7.5
FAK
4.4
2.4
GAPDH

FIGURE 1

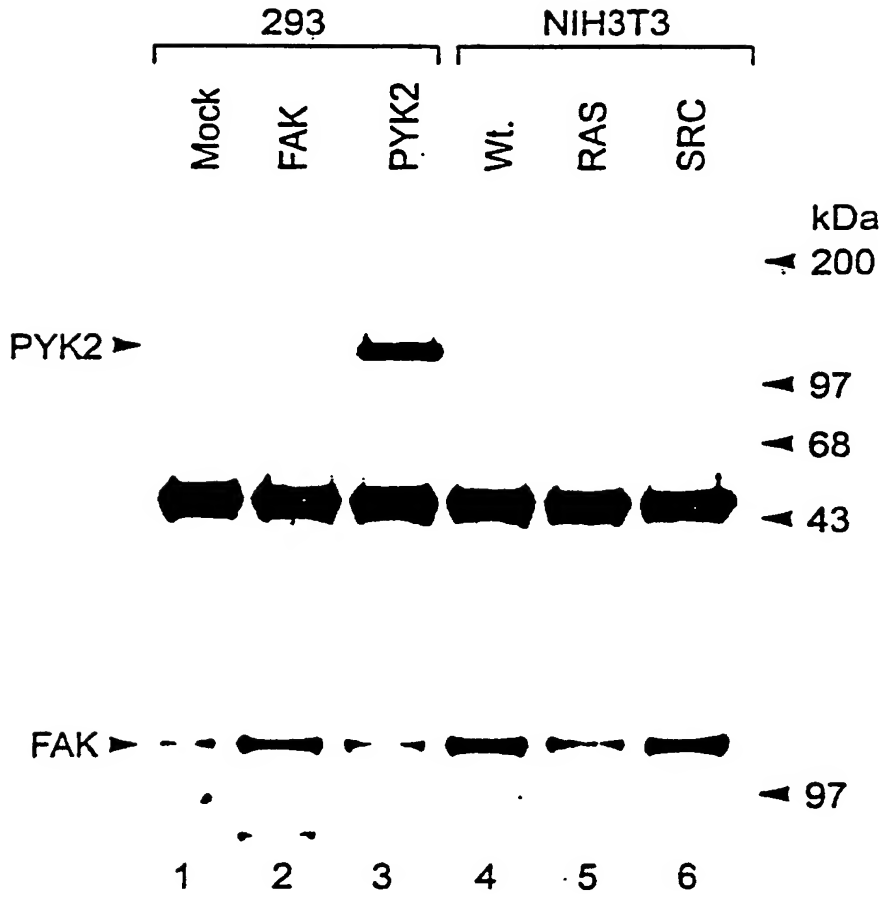

FIGURE 2

Figure 3

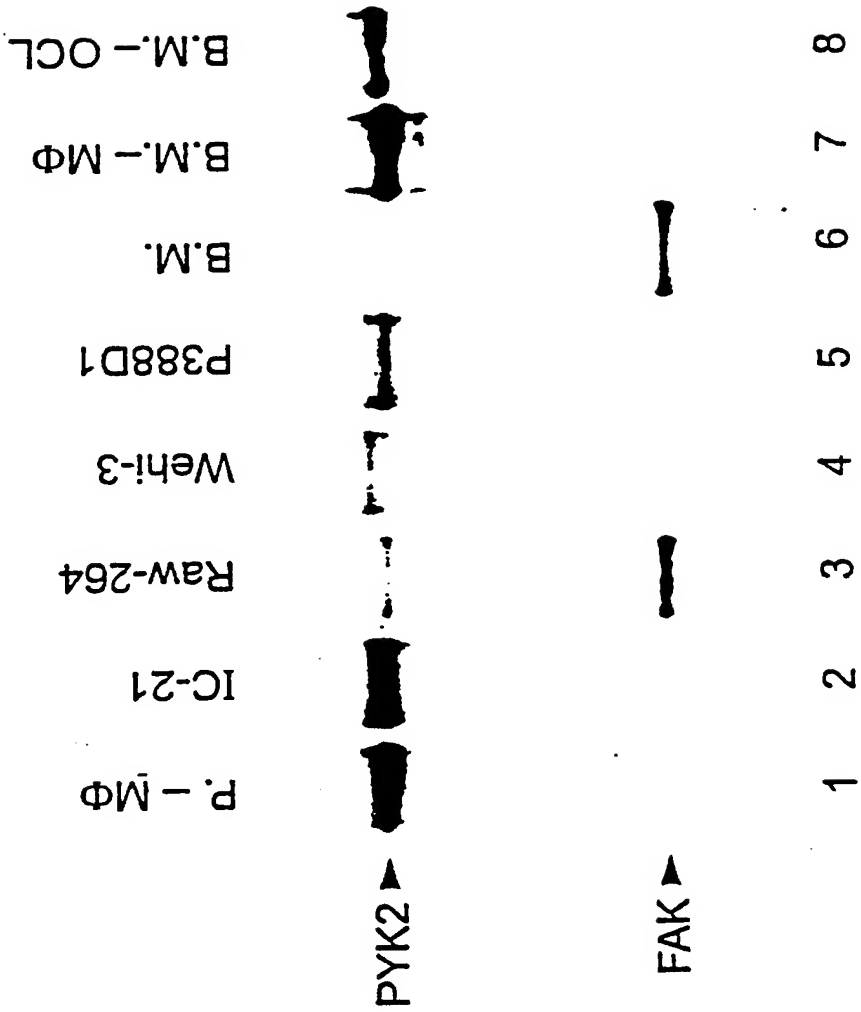

FIGURE 3

Figure 4

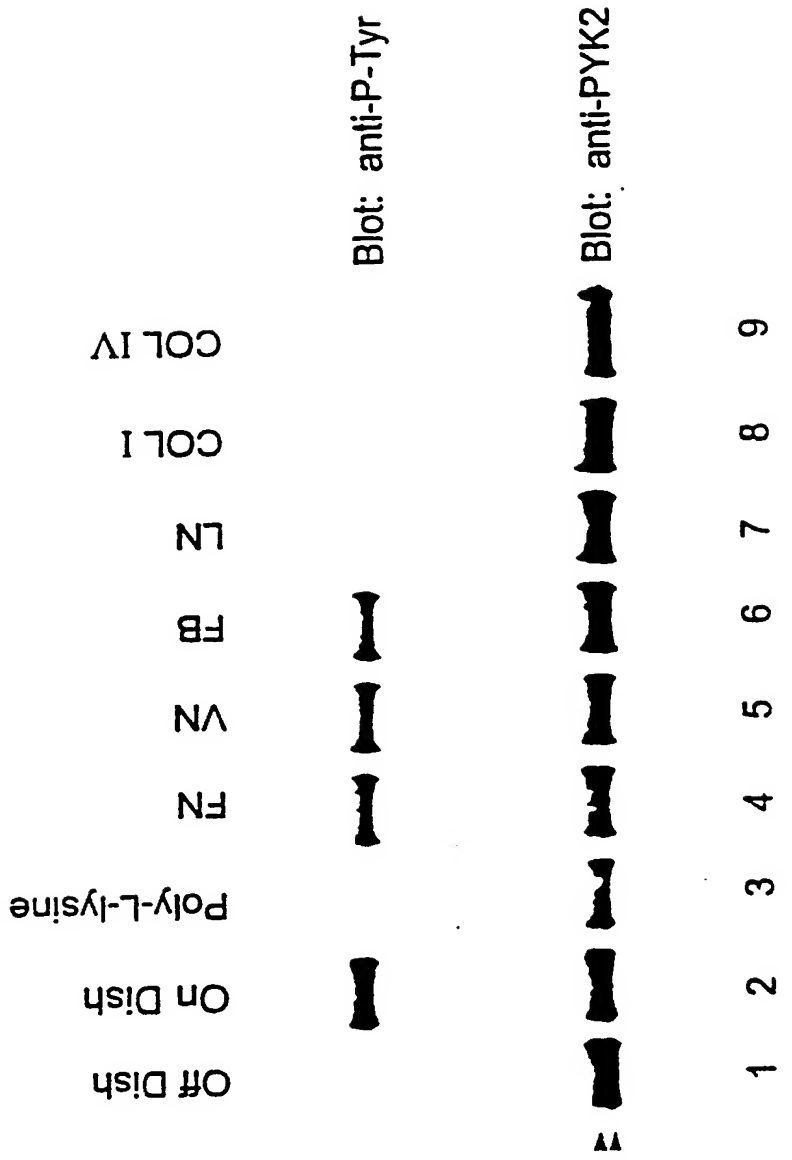

FIGURE 4

Figure 5A

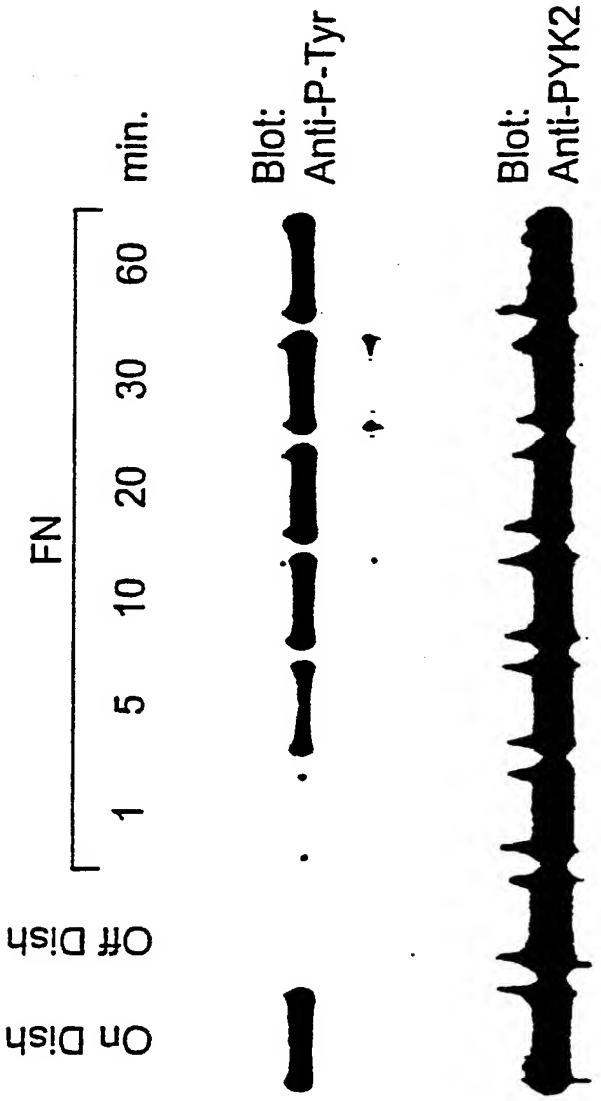

FIGURE 5A

Figure 5B

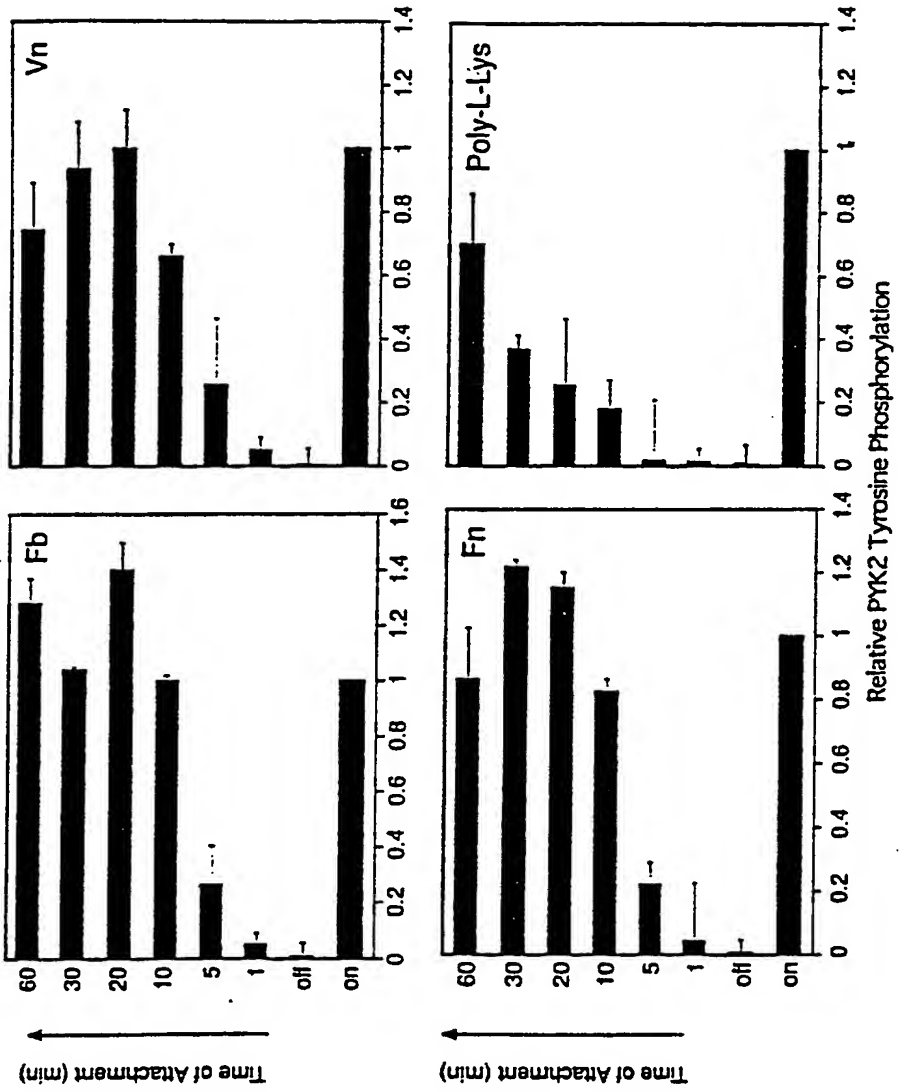

FIGURE 5B

Figure 5C

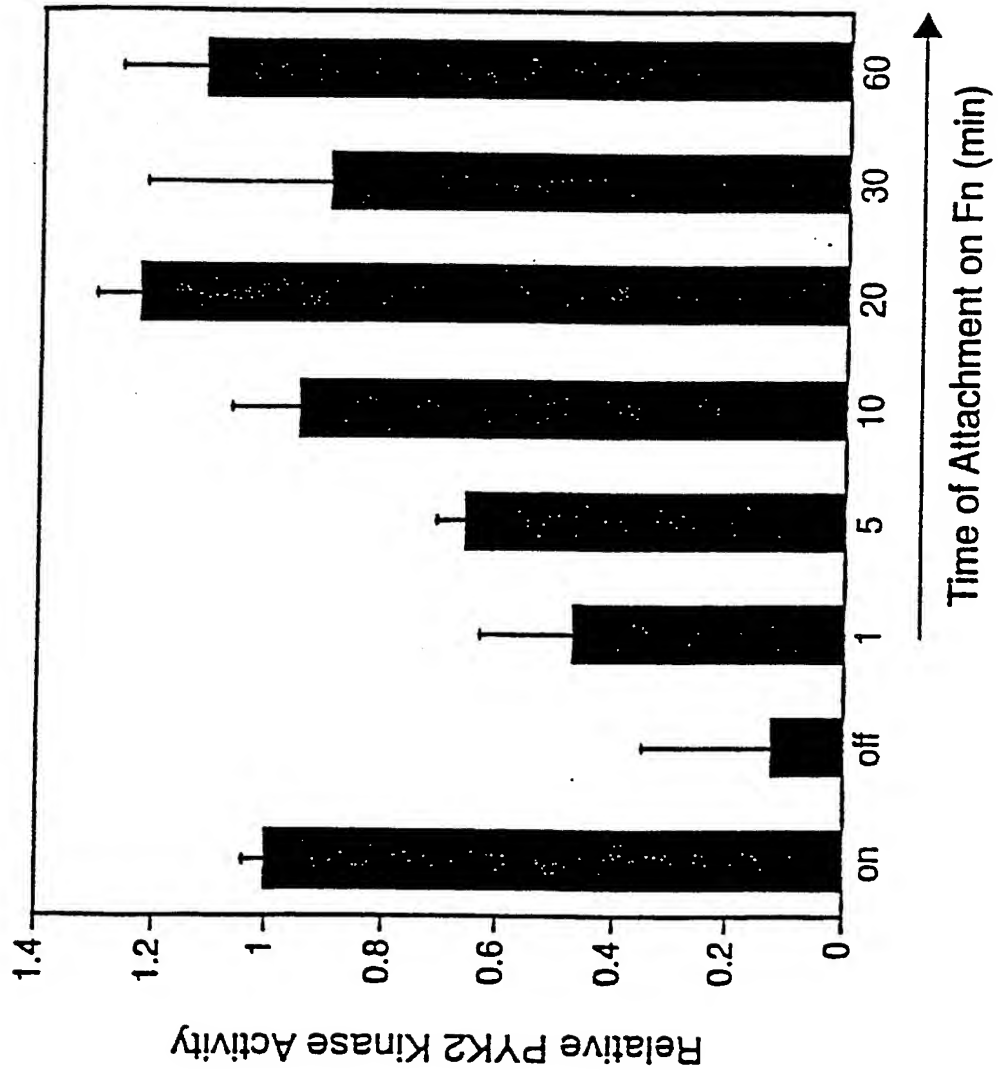

FIGURE 5C

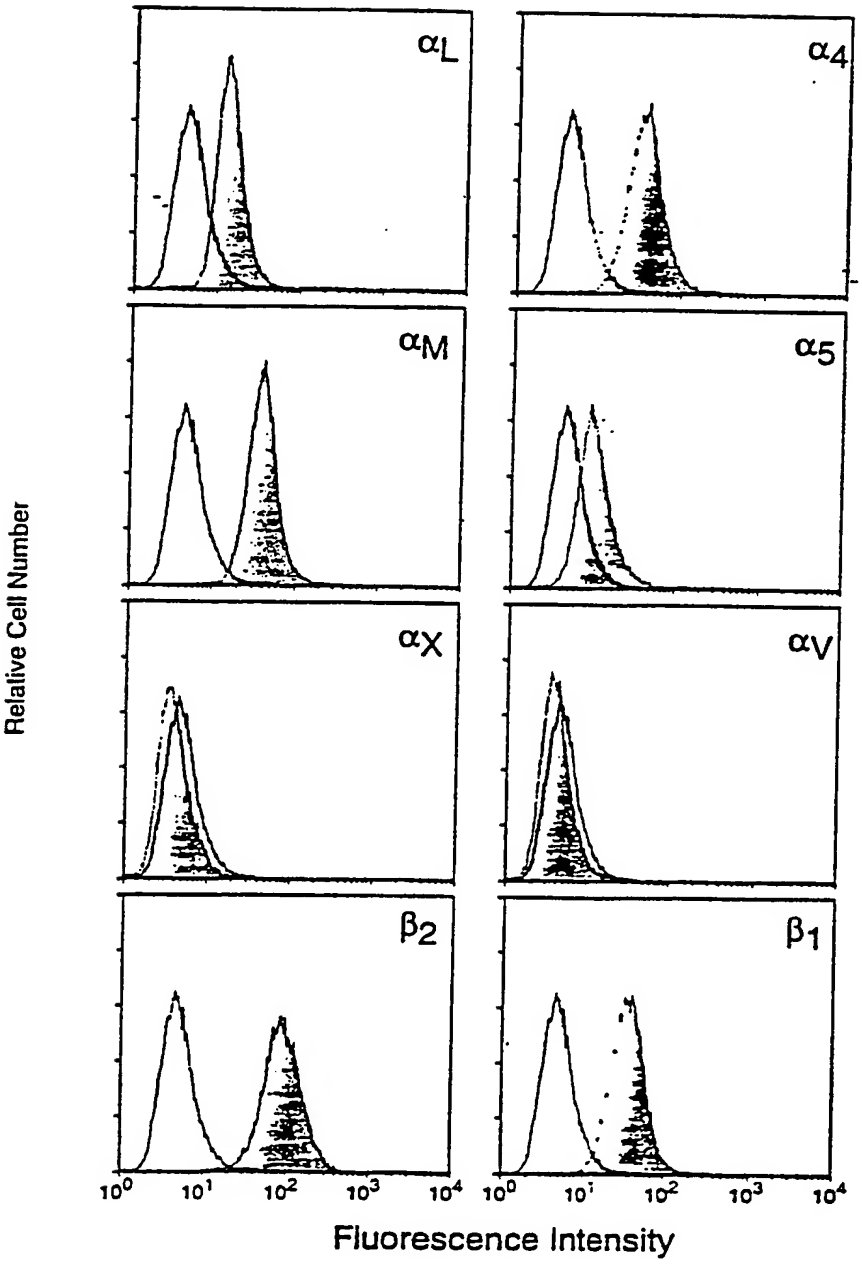

FIGURE 6A

Figure 6B

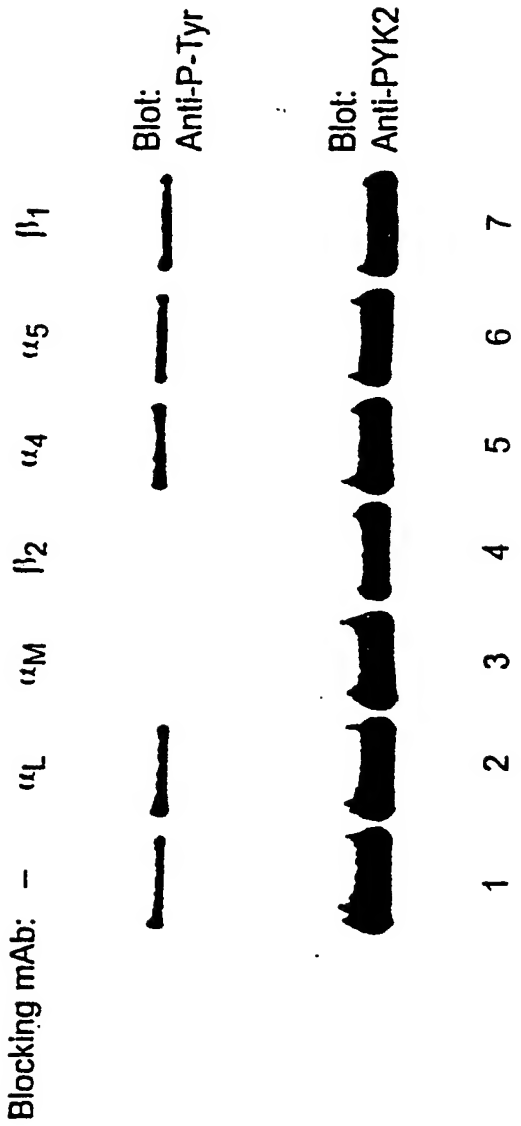

FIGURE 6B

Figure 6C

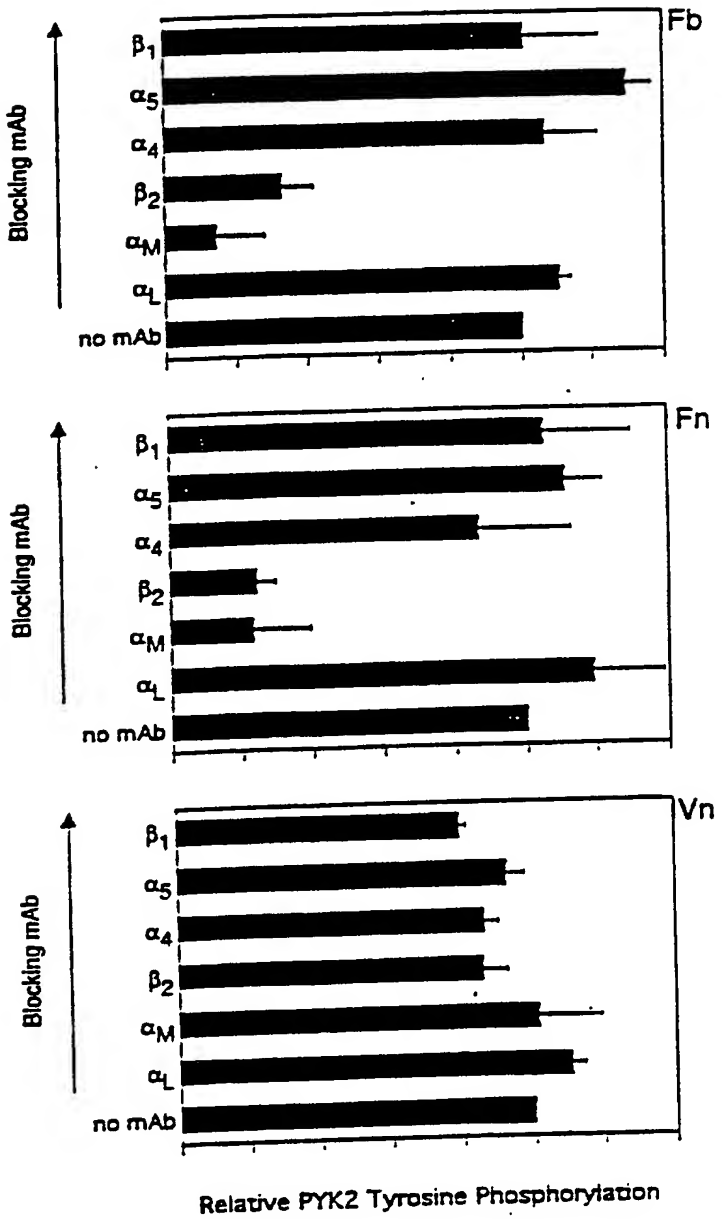

FIGURE 6C

Figure 7

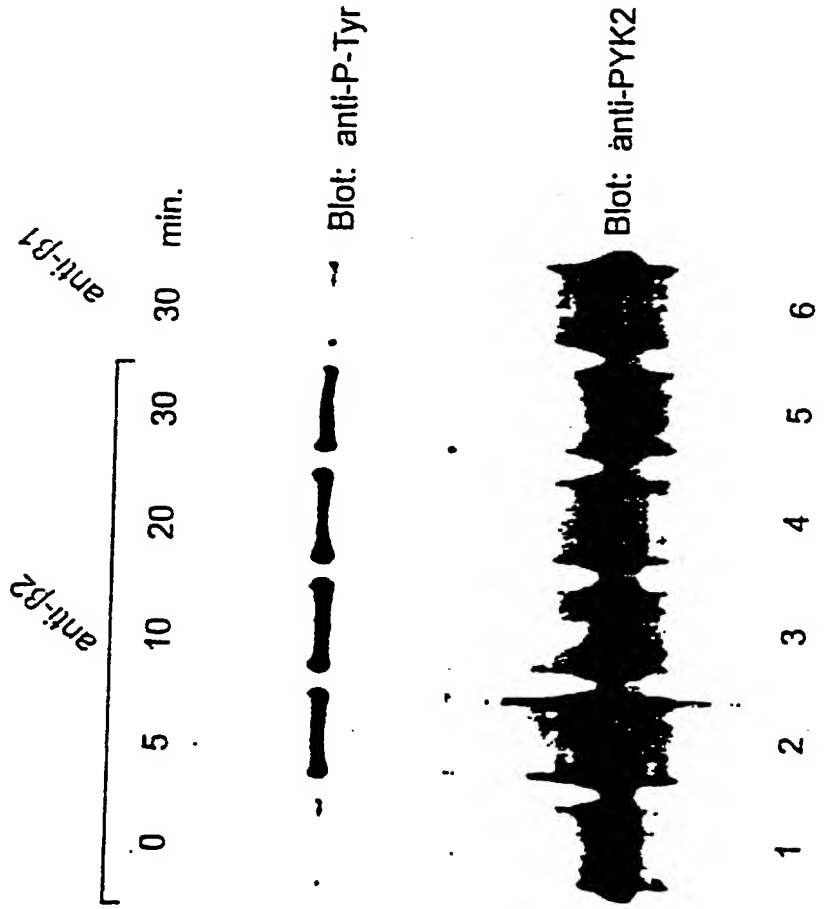

FIGURE 7

FIGURE 8

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  G  I  I  E  E  E  P  T  W  I  I  M  E  L  Y  P  Y  G  E  L     510
TGGGACACTACCTGGAACGAAATAAAAACTCCCTGAAGGTACCCACTCTGGTCCTGTACA    1740
  G  H  Y  L  E  R  N  K  N  S  L  K  V  P  T  L  V  L  Y  T     530
CCCTACAGATATGCAAAGCCATGGCCTATCTGGAGAGCATCAACTGTGTGCACAGGGATA    1800
  L  Q  I  C  K  A  M  A  Y  L  E  S  I  N  C  V  H  R  D  I     550
TTGCTGTCCGGAACATCCTGGTGGCCTCTCCTGAGTGTGTGAAGCTGGGGGACTTTGGGC    1860
  A  V  R  N  I  L  V  A  S  P  E  C  V  K  L  G  D  F  G  L     570
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FIGURE 8. ctd.

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TCTCCCGGTACATTGAGGACGAAGACTATTACAAAGCCTCTGTGACACGTCTACCCATCA  1920
 S  R  Y  I  E  D  E  D  Y  Y  K  A  S  V  T  R  L  P  I  K    590
AATGGATGTCCCCCGAGTCCATCAACTTCCGCCGCTTCACAACCGCCAGTGATGTCTGGA  1980
 W  M  S  P  E  S  I  N  F  R  R  F  T  T  A  S  D  V  W  M    610
TGTTTGCTGTATGCATGTGGGAGATCCTCAGCTTTGGGAAGCAGCCTTTCTTCTGGCTCG  2040
 F  A  V  C  M  W  E  I  L  S  F  G  K  Q  P  F  F  W  L  E    630
AAAATAAGGATGTCATCGGAGTGCTGGAGAAAGGGGACAGGCTGCCCAAGCCCGAACTCT  2100
 N  K  D  V  I  G  V  L  E  K  G  D  R  L  P  K  P  E  L  C    650
GTCCGCCTGTCCTTTACACACTCATGACTCGCTGCTGGGACTACGACCCCAGTGACCGGC  2160
 P  P  V  L  Y  T  L  M  T  R  C  W  D  Y  D  P  S  D  R  P    670
CCCGCTTCACGGAGCTTGTGTGCAGCCTCAGTGACATTTATCAGATGGAGAAGGACATTG  2220
 R  F  T  E  L  V  C  S  L  S  D  I  Y  Q  M  E  K  D  I  A    690
CCATAGAGCAAGAAAGGAATGCTCGCTACCGACCCCCTAAAATATTGGAGCCTACTACCT  2280
 I  E  Q  E  R  N  A  R  Y  R  Ⓟ  Ⓟ  K  I  L  E  Ⓟ  T  T  F    710
TTCAGGAACCCCCACCCAAGCCCAGCCGGCCCAAGTACAGACCTCCTCCACAGACCAACC  2340
 Q  E  Ⓟ  Ⓟ  Ⓟ  K  Ⓟ  S  R  Ⓟ  K  Y  R  Ⓟ  Ⓟ  Ⓟ  Q  T  N  L    730
TGCTGGCTCCTAAGCTGCAGTTCCAGGTCCCTGAGGGTCTGTGTGCCAGCTCTCCTACGC  2400
 L  A  Ⓟ  K  L  Q  F  Q  V  Ⓟ  E  G  L  C  A  S  S  Ⓟ  T  L    750
TTACCAGCCCTATGGAGTATCCATCTCCAGTTAACTCGCTGCACACCCCACCTCTCCACC  2460
 T  S  Ⓟ  M  E  Y  Ⓟ  S  Ⓟ  V  N  S  L  H  T  Ⓟ  Ⓟ  L  H  R    770
GGCACAATGTCTTCAAGCGCCACAGCATGCGGGAGGAGGACTTCATCCGGCCCAGTAGCC  2520
 H  N  V  F  K  R  H  S  M  R  E  E  D  F  I  R  P  S  S  R    790
GAGAAGAGGCCCAGCAGCTCTGGGAGGCAGAGAAGATCAAGATGAAGCAGGTCCTAGAAA  2580
 E  E  A  Q  Q  L  W  E  A  E  K  I  K  M  K  Q  V  L  E  R    810
GACAGCAGAAGCAGATGGTGGAAGATTCCCAGTGGCTGAGGCGAGAGGAAAGATGCTTGG  2640
 Q  Q  K  Q  M  V  E  D  S  Q  W  L  R  R  E  E  R  C  L  D    830
ACCCTATGGTTTATATGAATGACAAGTCCCCACTGACTCCAGAGAAGGAGGCCGGCTACA  2700
 P  M  V  Y  M  N  D  K  S  P  L  T  P  E  K  E  A  G  Y  T    850
CGGAGTTCACAGGGCCCCCACAGAAACCACCTCGGCTCGGTGCACAGTCCATTCAGCCCA  2760
 E  F  T  G  P  P  Q  K  P  P  R  L  G  A  Q  S  I  Q  P  T    870
CAGCCAACCTGGACAGGACCGATGACCTCGTGTACCACAATGTCATGACCCTGGTGGAGG  2820
 A  N  L  D  R  T  D  D  L  V  Y  H  N  V  M  T  L  V  E  A    890
CTGTGCTGGAACTCAAGAACAAGCTTGGCCAGTTGCCCCCTGAGGACTATGTGGTGGTGG  2880
 V  L  E  L  K  N  K  L  G  Q  L  P  P  E  D  Y  V  V  V  V    910
TGAAGAACGTGGGGCTGAACCTGCGGAAGCTCATCGGCAGTGTGGACGATCTCTTGCCCT  2940
 K  N  V  G  L  N  L  R  K  L  I  G  S  V  D  D  L  L  P  S    930
CCTTGCCGGCATCTTCGAGGACAGAGATTGAAGGGACCCAGAAACTGCTCAACAAAGACC  3000
 L  P  A  S  S  R  T  E  I  E  G  T  Q  K  L  L  N  K  D  L    950
TGGCAGAGCTCATCAACAAGATGAAGTTGGCTCAGCAGAACGCCGTGACGTCCCTGAGTG  3060
 A  E  L  I  N  K  M  K  L  A  Q  Q  N  A  V  T  S  L  S  E    970
AGGACTGCAAGCGGCAGATGCTCACAGCGTCCCATACCCTGGCTGTGGATGCCAAGAACC  3120
 D  C  K  R  Q  M  L  T  A  S  H  T  L  A  V  D  A  K  N  L    990
TGCTGGATGCTGTGGACCAAGCCAAGGTTGTGGCTAATCTGGCCCACCCGCCTGCAGAGt  3180
 L  D  A  V  D  Q  A  K  V  V  A  N  L  A  H  P  P  A  E  *
gatcaagagaggggccacctgcctgcatcttctgcccccacctgtcttggcatacctttc  3240
ctgccttgcctttggttattggtcttccagggaaagctgagaagagtccatccccccttgc  3300
cactttgcacgacacccccctcttcccccaacccacccccagactgtgctactcaggctgca  3360
tctggacagaaaggactctgggcacagacacggggtggggtgacatagttcatagggggta  3420
ctactgccagccactccctcttaccccagcctgggttgctggagcatcattggggtcatg  3480
agtgtacccctaacggccaagatggctttctgcatggacatttgagagccagtattcctc  3540
cttcctcttcagccctcagggacccctgatacagaggggacagagaggggttttatttgt  3600
agagaagctggtgagatgagggctggacctggctctcttgtacagtgtacattggaattt  3660
atttaatgtgagtttgacctggatggacagccaagggccatagtccaggagcaaaccaat  3720
ccagtcacaggactctgtgttttatggaactgagtgccacaggaagaagcagagagtcgg  3780
aggtcagaatggactttgtgcccttcctgcgtttctcttctccctctttccttctcccct  3840
cttttcttacgtctccttttttctcctccccctttcacatctgctcccctcctctctcat  3900
gtctgtggagaacatttaccttccttcttttttgatcggtggttgaattaaaattattacc  3960
atttgctttgtggctcgtgcc                                          3981
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02797

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/48; C12N 9/12, 15/52; C07K 14/00; A61K 38/17
US CL :435/7.1, 15, 69.1, 194; 514/2; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 15, 69.1, 194; 514/2; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LEV et al. Protein Tyrosine Kinase PYK2 Involved in Ca^{+2}-Induced Regulation of Ion Channel and MAP Kinase Functions. Nature. 31 August 1995, Vol. 376, pages 737-745, especially pages 738-741.	1-22
Y	SASAKI et al. Cloning and Characterization of Cell Adhesion Kinase β, a Novel Protein-Tyrosine Kinase of the Focal Adhesion Kinase Subfamily. The Journal of Biological Chemistry. 08 September 1995, Vol. 270, No. 36, pages 21206-21219, see especially pages 21216-21219.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 03 APRIL 1998	Date of mailing of the international search report 18 JUN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SALLY P. TENG Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Internati nal application No.
PCT/US98/02797

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHLAEPFER et al . Signal Transduction from the Extracellular Matrix--A Role for the Focal Adhesion Protein-Tyrosine Kinase FAK. Cell Structure and Function. October 1996, Vol. 21, No. 5, pages 445-450, especially pages 446-448.	1-22
Y	SICILIANO et al. Differential Regulation of Proline-Rich Tyrosine Kinase 2/Cell Adhesion Kinase β (PYK2/CAKβ) and pp125^{FAK} by Glutamate and Depolarization in Rat Hippocampus. The Journal of Biological Chemistry. 15 November 1996, Vol. 271, No. 46, pages 28942 to 28946, especially pages 28944-28946.	1-22
Y	SEUFFERLEIN et al. Dissociation of Mitogen-Activated Protein Kinase Activation from p125 Focal Adhesion Kinase Tyrosine Phosphorylation in Swiss 3T3 Cells Stimulated by Bombesin, Lysophosphatidic Acid, and Platelet-Derived Growth Factor. Molecular Cell Biology. December 1996, Vol. 7, pages 1865-1875, especially pages 1867-1872.	1-22
Y	WEBER et al. Inhibitors of Protein Tyosine Kinase Suppress TNF-Stimulated Induction of Endothelial Cell Adhesion Molecules. The Journal of Immunology. 01 July 1995, Vol. 155, No. 1, pages 445-451, especially pages 446-449.	1-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02797

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02797

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Biosis, Medline, WPI
search terms: protein tyrosine kinase, cell adhesion kinase, related adhesion focal adhesion kinase, monocyte adhesion, osteoclast mobility, inhibitor, extracellular matrix, osteoporosis, treatment.